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Phosphorylation of the N-terminal domain of *Xenopus* TATA-box binding protein by DNA-dependent protein kinase depends on the C-terminal core domain

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Abstract DNA-dependent protein kinase (DNA-PK) has been shown to phosphorylate several transcription factors in vitro, suggesting that this nuclear enzyme – in addition to its role in DNA repair and recombination – may be involved in transcriptional regulation. In the typical mechanism the DNA-bound kinase phosphorylates a substrate that is bound to the same DNA molecule. Here I report that the *Xenopus* TATA-box binding protein (xTBP) is hyperphosphorylated by DNA-PK in vitro. The phosphorylation is in the N-terminal domain of the protein but depends fully on the presence of the C-terminal core domain.

Key words: DNA-dependent protein kinase; TATA-box binding protein; *Xenopus laevis*

1. Introduction

DNA-dependent protein kinase (DNA-PK) is an abundant nuclear serine/threonine protein kinase comprised of a 460-kDa catalytic subunit and the 70/80-kDa Ku heterodimer [1–3]. The Ku proteins interact with double-stranded DNA and this interaction activates the catalytic subunit, which phosphorylates many proteins involved in nuclear processes like transcription, DNA replication and recombination [4-6]. Recent studies indicated that DNA-PK is involved in signaling pathways related to DNA repair and recombination, but its in vivo substrates in these processes have not been determined [7,8]. On the other hand, a physiological role for DNA-PK in transcription has not been proven so far, but is strongly suggested by the fact that many transcription factors are substrates for DNA-PK. It has been difficult, however, to demonstrate an effect on the function of these proteins upon phosphorylation by DNA-PK.

The TATA-box binding protein (TBP) is a highly conserved eukaryotic transcription factor that plays a central role in the assembly of initiation complexes on promoters for all three classes of nuclear RNA polymerase (pol) [9,10]. Little is known about the phosphorylation of TBP and nothing about the significance of this modification. TBP was reported to be hyperphosphorylated during mitosis, but the protein kinase responsible for this modification has not been identified [11]. Furthermore, the TFIIH-associated kinase was found to phosphorylate the N-terminal domain of human TBP in vitro [12]. The present report adds to our knowledge of TBP phosphorylation by showing that *Xenopus* TBP (xTBP) is phosphorylated by DNA-PK on multiple sites in its variable N-terminal domain and that this hyperphosphorylation is dependent on the evolutionarily conserved C-terminal domain.

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2. Materials and methods

A pGEX-2T plasmid carrying the cDNA for xTBP was a gift from A. Leresche and J. Gottesfeld (La Jolla). The xTBP cDNA had been generated by polymerase chain reaction (PCR) amplification from *Xenopus* cDNA using primers derived from the sequence deposited in Genbank [13]. The *Bam*HI-*Eco*RI insert was excised and cloned into the pET-28a(+) expression vector (Novagen) to give rise to the plasmid pET-xTBP. The N- and C-terminal deletions were synthesized in vitro by PCR with Pfu DNA polymerase (Stratagene) using linear pET-xTBP as template and appropriate oligonucleotide primers. The resulting constructs were verified by DNA sequencing. Four nucleotide changes were observed when compared to the sequence in Genbank: $A_{164} \rightarrow G$, $A_{390} \rightarrow G$, $G_{531} \rightarrow A$, and $A_{534} \rightarrow G$. Two of these nucleotide changes resulted in amino acid changes: $Q_{55} \rightarrow R$, and $M_{177} \rightarrow I$. The same two amino acid positions were found to be changed in two independent clones of the original pGEX-2T construct and might represent allelic or animal variation. They do not affect DNA-binding and transcriptional function of the expressed xTBP protein (J. Gottesfeld, personal communication).

pET-xTBP and its deletions were transferred to *E. coli* BL21(DE3) and expression of the hexahistidine-tagged fusion proteins was induced for 4 h by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside during logarithmic growth. Bacterial pellets were resuspended in lysis buffer (0.3 M NaCl, 50 mM Na-phosphate [pH 8], 1 mM phenylmethanesulfonyl fluoride) containing 5 mM $MgCl_2$, 0.1% Triton X-100, 40 mM imidazole, 1 mg/ml lysozyme, 10 mM benzamidine-HCl, 1 mg/ml pepstatin, 2 mg/ml aprotinin, and 1–5 μg/ml DNase I. After 10 min at room temperature, the cell suspension was sonicated for 10 min at 4°C in a Branson 1200 sonicator. The lysate was centrifuged for 1 h at 30000 rpm in a Beckman SW60 rotor. The cleared lysate was applied to an Ni-NTA Agarose column (Qiagen) equilibrated in a lysis buffer containing 40 mM imidazole. After rinsing the column with lysis buffer containing 60 mM imidazole, the his-tagged fusion protein was eluted with lysis buffer containing 250 mM imidazole. Fractions containing his-tagged fusion protein were pooled and dialyzed against 100 vols. of 0.1 M KCl, 20 mM HEPES, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 0.5 mM PMSF for 16 h.

A typical kinase reaction consisted of 25 mM Tris-HCl (pH 8), 6.25 mM $MgCl_2$, 50 mM KCl, 0.05% NP-40, 0.5 mM EDTA, 10% glycerol, 5 mM DTT, 0.2 mM [γ-^{32}P]ATP (5 Ci/mmol), 200 ng substrate protein, linear double-stranded DNA, and 0.25 units DNA-PK (Promega) in a volume of 20 μl. After 30 min at room temperature, the reaction was stopped by adding 30 μl of 2×protein gel loading buffer. Heat-denatured samples were analyzed on 12 or 15% discontinuous SDS-polyacrylamide gels [14]. The DNA in the reaction was either a 3585-bp plasmid linearized with *Ssp*I (similar to pMH-T3wt in [15]), a 161-bp *Hin*dIII-*Eco*RI fragment containing *X. laevis* ribosomal DNA (nucleotides +1 to +158 [16]), or a 124-bp *Hin*dIII-*Eco*RI fragment containing 3 copies of the 26-bp sequence GATCGGGGGCTA-TAAAAGGGGGTGGG cloned as head-to-tail repeats into the *Bam*HI site of pGem4.

Prior to endoproteinase digestion, the reaction mixture was incubated at 65°C for 15 min to inactivate DNA-PK. For digestion with thrombin (Sigma), 1 μl of 50 mM $CaCl_2$ and 1 μl of thrombin (125

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ng/μl) was added and the reaction mixture was incubated at room temperature for 1 h. For digestion with Glu-C and Lys-C (Promega), one half of the kinase reaction mixture was diluted 8-fold with either 100 mM ammonium acetate (pH 4), or with 25 mM Tris-HCl (pH 7.6), 1 mM EDTA, respectively, and 600 ng endoproteinase was added. Digestion was for 5 h at room temperature. While thrombin cleaved his-xTBP quantitatively in 5 min, complete digestion with Glu-C and Lys-C could not be achieved even with more enzyme or at 37°C (data not shown). Endoproteinase digests were analyzed on 16.5% discontinuous Tricine-SDS-polyacrylamide gels [17].

3. Results

Recombinant hexahistidine-tagged xTBP (his-xTBP) was incubated in vitro with purified DNA-PK and linear plasmid DNA in the presence of [γ-^{32}P]ATP. The reactions were analyzed by protein gel electrophoresis and autoradiography. Fig. 1 (lanes 1–7) shows that his-xTBP was phosphorylated in a DNA-dependent manner. The low basal level of phosphorylation in the absence of added DNA (lane 1) was drastically increased in the presence of 1–10 ng (50–500 ng/ml) of linear plasmid DNA (lanes 2–4). Phosphorylation decreased with more DNA (lanes 5–7) and was reduced almost to the level of the no-DNA control with 100 ng of DNA (lane 7). Control reactions with varying amounts of circular plasmid DNA showed only basal level of phosphorylation (data not shown). Optimal phosphorylation of DNA-binding proteins at low concentrations of linear DNA was previously observed [2,18] and interpreted to indicate a requirement for colocalization of substrate and kinase on the same DNA fragment. Phosphorylation of his-xTBP resulted in at least four forms having different electrophoretic mobilities, indicating that individual xTBP molecules were phosphorylated on multiple sites. Staining of the gels with Coomassie blue before autoradiography revealed that the fastest form migrated with the input nonphosphorylated protein (data not shown). Interestingly, the mobility-shifted forms were not seen upon staining, indicating that the bulk of his-xTBP in the reaction remained nonphosphorylated and that hyperphosphorylation occurred cooperatively. While it is currently not clear why xTBP was not quantitatively phosphorylated in the present reactions, this fact should not affect any of the conclusions drawn. As discussed previously [4], the rate of phosphorylation of DNA-binding substrates by DNA-PK may depend on several factors, including the on- and off-rates for DNA-binding of the substrate.

In the reactions shown in Fig. 1 (lanes 1–7) the DNA activating the kinase did not contain a true eukaryotic TATA-box, but plasmid DNA contains TATA-box like sequences in prokaryotic promoters. To further investigate the sequence requirement for the phosphorylation of his-xTBP by DNA-PK, I compared a restriction fragment containing *X. laevis* rDNA (GC content 85%) with a fragment containing three tandem repeats of the Adenovirus major late promoter TATA-box with respect to their ability to induce phosphorylation of his-xTBP by DNA-PK. Fig. 1 (lanes 8–16) shows that both DNA fragments were equally effective in activating the phosphorylation of his-xTBP by DNA-PK. The present his-xTBP was found to form specific complexes with the TATA-box in electrophoretic mobility shift assays (data not shown), but the observed lack of sequence specificity in DNA-PK reactions might be related to the fact that TBP also interacts with non-cognate DNA sequences [19].

To identify the region on the xTBP protein that is phosphorylated by DNA-PK, phosphorylated his-xTBP was digested with Glu-C and Lys-C endoproteinases and analyzed on Tricine-SDS gels. Digestion of his-xTBP with Glu-C was expected to yield products of 2.2, 3.6, 3.8 and 5.0 kDa (plus some smaller polypeptides) derived from the core domain, and fragments of 6.6 and 9.0 kDa from the N-terminal domain, all of which would have been resolved on the gel used. Digestion with Lys-C would generate a 17.7-kDa fragment encompassing the entire N-terminal domain plus the his-tag, while the core domain would be cut into several small fragments plus a 2.6- and a 3.8-kDa product (Fig. 2, top) (note that in Fig. 2 the size of these digestion products is given in numbers of amino acid residues). The autoradiograph in Fig. 2 (bottom) shows that the major phosphorylated Glu-C digestion product migrated at about 8–10 kDa (lane 3), while Lys-C digestion resulted in a broad band running at about 20 kDa (lane 4). It is important to note that these major digestion products con-

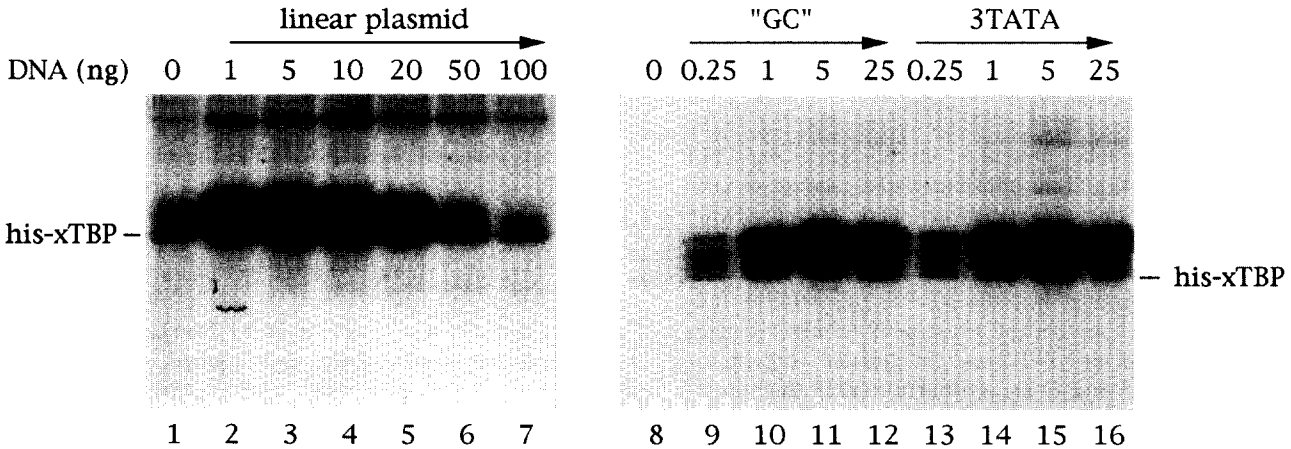

Fig. 1. Phosphorylation of his-xTBP by DNA-PK in vitro. Kinase reactions were carried out in the presence of a 3.6-kb plasmid linearized with *Ssp*I (lanes 2–7), or in the presence of *Hin*dIII-*Eco*RI fragments containing either GC-rich *X. laevis* ribosomal DNA (lanes 9–12) or three copies of a 26-bp sequence containing a TATA-box (lanes 13–16). The amount of DNA added to the 20-μl reactions is indicated above the lanes. An autoradiograph of ^{32}P-labeled proteins is shown; the migration of the bulk of the nonphosphorylated his-xTBP is indicated. The basal level of phosphorylation without added DNA in the two panels (lanes 1,8) is different because the autoradiographs are from two independent experiments.

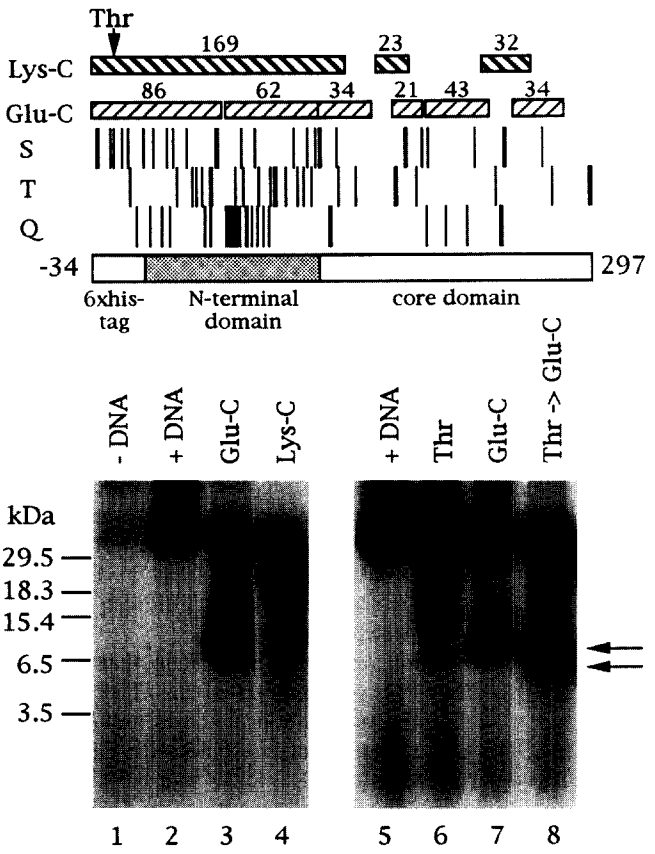

Fig. 2. Endoproteinase mapping of the region of his-xTBP phosphorylated by DNA-PK. (Top) Map of his-xTBP indicating the hexahistidine tag (amino acid residues −34 to −1), the N-terminal domain (residues +1 to +116) and the conserved core domain (residues +117 to +297). Above the map, the locations of serine (S), threonine (T), and glutamine (Q) residues, the predicted digestion products with Lys-C and Glu-C, as well as the position of the thrombin (Thr) cleavage site are shown. The lengths of the Lys-C and Glu-C fragments are given in numbers of amino acids. Only fragments larger than 20 amino acids are shown. (Bottom) Autoradiograph of a Tricine-SDS-protein gel showing control reactions without (lane 1) and with (lanes 2,5) DNA and digestions of reactions as in lanes 2,5 with the endoproteinases indicated above the lanes (lanes 3,4,6–8). The approximate migration of marker proteins is shown to the left. The two arrows point to the ^{32}P-labeled 86-residue Glu-C fragment (lane 7) and its thrombin digestion product (lane 8). The slow-migrating strong bands in lanes 7,8 are due to incomplete digestion with Glu-C in these reactions (compare to the more complete digestion in lane 3).

tained most of the label present in the undigested his-xTBP (compare lanes 2–4) and no phosphorylated products smaller than the Glu-C fragment were detected. Thus, both the Glu-C and Lys-C digestion products indicated that DNA-PK phosphorylated the N-terminal domain. To decide whether the Glu-C digestion product was the 6.6- or the 9.0-kDa fragment, both of which are derived from the N-terminal domain, the phosphorylated his-xTBP was first digested with thrombin. Fig. 2 (lanes 5 and 6) shows that thrombin digestion – as expected – did not detectably change the mobility of the labeled his-xTBP on this high percentage polyacrylamide gel, and only a few non-specific digestion products were observed. Analysis of the same samples on a lower percentage polyacrylamide gel showed that thrombin quantitatively cleaved off the 1.9-kDa hexahistidine tag (not shown). The control and thrombin-digested his-xTBP were then further digested with Glu-C (lanes 7,8). It can be seen that thrombin reduced the molecular weight of the Glu-C fragment, demonstrating that the ^{32}P-labeled fragment was the most N-terminal 9.0-kDa fragment. The endoproteinase mapping experiments thus indicate that most, if not all, of the phosphorylation by DNA-PK takes place in the very N-terminus of his-xTBP.

The conserved core domain of TBP is known to contain the DNA-binding domain [20,21]. To investigate whether the core domain was required for phosphorylation of the N-terminal domain by DNA-PK, the following deletions of his-xTBP were constructed (Fig. 3; top): a partial deletion of the N-terminal domain (xTBPΔ1/2N), a complete deletion of the N-terminal domain (xTBPΔN), and a complete deletion of the C-terminal core domain (xTBPΔC). Fig. 3 (lanes 1–4) shows the full-length his-xTBP and the deleted xTBP proteins on a Coomassie blue-stained gel. Fig. 3 (lanes 5–12) shows the phosphorylation of these constructs by DNA-PK in the absence and presence of 10 ng linear plasmid DNA. Interestingly, complete deletion of the segment that is most heavily phosphorylated in the full-length construct (as determined by the endoproteinase mapping experiments), caused only a small (up to 2-fold) reduction in DNA-dependent phosphorylation (lanes 7,8). Similar to the full-length protein, phosphorylated TBPΔ1/2N showed at least three species of different mobilities. This result indicates that upon deletion of the distal half of the N-terminal domain, phosphorylation now shifts to the proximal part of the N-terminal domain. Complete removal of the N-terminal domain virtually abolished phosphorylation by DNA-PK, and the remaining low level of phosphorylation was not activated by 10 ng DNA (lanes 9,10). However, the N-terminal domain by itself (lanes 11,12) showed even less phosphorylation by DNA-PK (the position of TBPΔC as determined by a longer exposure and by Coomassie blue staining is indicated by the arrow).

It has been found that the optimal DNA concentration for phosphorylation by DNA-PK is different for non-DNA-binding and for DNA-binding substrates [4,18]. I therefore investigated the effect of 200 ng (10 μg/ml) DNA on phosphorylation of the isolated N- and C-terminal domains. Again, the full-length his-xTBP was much less phosphorylated in the presence of 200 ng linear DNA than with 10 ng DNA (lanes 13–15), while the core domain TBPΔN by itself showed no DNA-dependent phosphorylation with 10 ng DNA (lanes 16,17). Interestingly, some DNA-dependent phosphorylation of TBPΔN was induced with 200 ng DNA, and in addition to the major labeled form a small amount of a slower-migrating form was detected (lane 18). While the phosphorylation of TBPΔN was only ~10% of the maximal level achieved with the full-length xTBP, it confirmed that DNA-PK was still active under these conditions [4]. Most importantly, the isolated N-terminal domain showed no significant DNA-dependent phosphorylation at any of the DNA concentrations tested (lanes 19–21). The combined results show that even though hyperphosphorylation of full-length xTBP takes place exclusively in the N-terminal domain, this phosphorylation strictly depends on the core domain.

4. Discussion

In this study I have analyzed the phosphorylation of xTBP by DNA-PK. The reaction appears to follow the paradigm of the mechanism by which DNA-PK phosphorylates its sub-

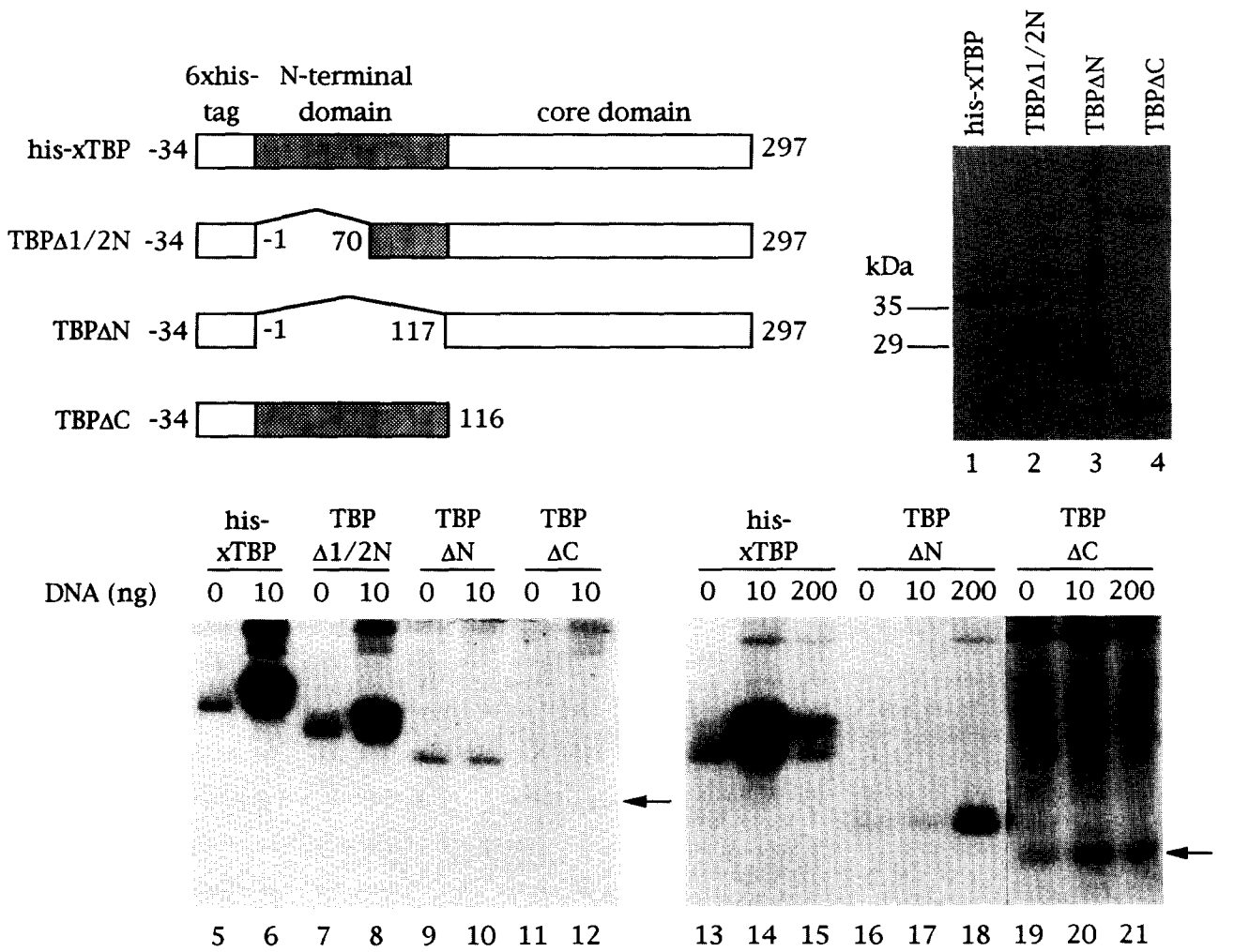

Fig. 3. Phosphorylation of deleted his-xTBP constructs by DNA-PK. (Top left) Structure of the his-xTBP constructs. The numbers of the first and last amino acid residues of undeleted portions of the constructs are given. The first amino acid of the xTBP sequence is denoted residue +1. (Top right) Protein gel stained with Coomassie blue showing 2 μg each of full-length his-xTBP (lane 1) and the deleted constructs (lanes 2–4). The predicted molecular weights of these proteins are 36.2, 28.8, 23.9, and 15.8 kDa. (Bottom) Phosphorylation of the various constructs by DNA-PK in the presence of linear plasmid DNA. The type of substrate (200 ng each) and the amount of DNA in each of the reactions is given above the lanes. The reactions analyzed in lanes 5–12 and 13–21 are from two independent experiments. For lanes 19–21, an approx. 8-times longer exposure is shown. Note that the isolated N-terminal domain (TBPΔC, lanes 11,12,19–21; position indicated by arrows) shows only basal level phosphorylation by DNA-PK, while the isolated core domain (TBPΔN, lanes 9,10,16–18) is only significantly phosphorylated in the presence of 200 ng DNA (lane 18).

strate. In this mechanism, DNA-PK binds non-specifically to DNA and phosphorylates a protein that is bound via its DNA-binding domain to its cognate recognition sequence on the same DNA molecule. The phosphorylation of Sp1, c-jun and the C-terminal domain (CTD) of the largest subunit of pol II (with a promoter being the recognition sequence) by DNA-PK are examples where evidence for such a mechanism was presented [6,22-24]. In many cases, however, no requirement for the recognition sequence for the substrate in the activating DNA was found. Similar to the observation reported here for xTBP, serum response factor and p53 were phosphorylated equally well by DNA-PK whether or not the activating DNA contained the cognate binding site [4,25]. At least in the case of DNA-binding substrates, the requirement for the DNA-binding domain of the protein appears to be more stringent. Thus, similarly as reported here for xTBP, phosphorylation by DNA-PK of Sp1 and a GAL4-CTD fusion protein depended on the DNA-binding domain [22,26]. However, while the present data suggest a requirement for the C-terminal domain to target xTBP to DNA, they do not rule out models in which the C-terminal domain mediates phosphorylation of the N-terminal domain by changing the conformation of the entire protein or by binding to DNA-PK.

The preferred sequence motif for DNA-PK was identified to be S/T-Q, but it is clear that DNA-PK also phosphorylates other sites [4,18,24]. xTBP has only one T-Q and no S-Q motif [13]. The N-terminal domain is overall rich in S, T, and Q residues, however (see Fig. 2). It has been pointed out that the S- and T-containing motifs in the N-terminal domain of human TBP are reminiscent of the heptapeptide repeats in the CTD of pol II [27]. Like xTBP, the CTD does not have S/T-Q motifs but is nevertheless a good substrate for DNA-PK [23,26]. It is therefore tempting to speculate that DNA-PK recognizes similar elements in both the N-terminal domain of xTBP and in the CTD of pol II.

Even though the N-terminal domain of TBP is nonconserved if compared among all species, it contains regions that are highly conserved between human, mouse, snake and

Xenopus [13,28]. A search of the Genbank database for sequences with high similarity to the *Xenopus* N-terminal domain retrieved all these vertebrate TBP sequences, but none of the known non-vertebrate TBP sequences. All the vertebrate N-terminal domains differ mainly in the length of a Q stretch located near the center of the domain, while the segment from residue 1 to 54 in xTBP is 80–82% identical to that of the other vertebrate TBPs, and the segment from residue 73 to 116 is even 91% identical. Thus, it is to be expected that the N-terminal domain of all vertebrate TBPs can be phosphorylated by DNA-PK. Indeed, unpublished experiments (S.P. Lees-Miller and C.W. Anderson, personal communication) showed that human TBP is also hyperphosphorylated by DNA-PK and that the phosphorylation is much reduced upon deletion of the N-terminal domain. Furthermore, these investigators found no significant phosphorylation of yeast TBP, whose N-terminal domain shows no sequence similarity to the vertebrate N-terminal domains.

The precise function of the N-terminal domain of TBP remains unknown. Clearly, this domain is not required for basic promoter function and for the assembly with pol I-, pol II- and pol III-specific TBP-associated factors (TAFs) [29-31]. However, while some studies found that the N-terminal domain is also dispensable for activated transcription [32], others reported a requirement for the N-terminal domain for activated pol II transcription and for transcription from TATA-containing pol III promoters [33,34]. Furthermore, the N-terminal domain inhibits DNA binding in vitro [35], and a recent study suggested a role for the N-terminal domain in mediating protein-protein interactions [36]. Thus, an involvement in certain transcriptional regulatory mechanisms is very likely, and phosphorylation of the N-terminal domain by DNA-PK might affect interaction of TBP with both DNA and proteins.

Finally, I would like to discuss the present data in the light of the recent discovery that DNA-PK represses transcription by pol I [37,38]. To date, ribosomal gene transcription is the only cellular process shown to be sensitive to phosphorylation by DNA-PK in vitro. Kuhn et al. [37] showed that phosphorylation of the TIF-IB fraction by DNA-PK decreased transcription initiation at the mouse ribosomal gene promoter. Using a similar experimental protocol, I found that pretreatment of a chromatographic fraction containing the *Xenopus* pol I factor Rib1 with DNA-PK inhibited pol I transcription [38]. TIF-IB has been shown to consist of TBP and 3 pol I-specific TAFs [39]. Rib1 appears to be its *Xenopus* equivalent but has not been molecularly characterized [40]. These observations raised the possibility that DNA-PK phosphorylates and inactivates a polypeptide in the promoter-bound TBP-TAF complex. Future experiments will address the question whether the repression of pol I transcription by DNA-PK is due to phosphorylation of TBP.

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